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Conserved nucleoprotein structure at the ends of vertebrate and invertebrate chromosomes

(nucleosome/telomere/micrococcal nuclease)

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ABSTRACT **Eukaryotic chromosomes terminate with telomeres, nucleoprotein structures that are essential for chromosome stability. Vertebrate telomeres consist of terminal DNA tracts of sequence $(TTAGGG)_n$, which in rat are predominantly organized into nucleosomes regularly spaced by 157 bp. To test the hypothesis that telomeres of other animals have nucleosomes, we compared telomeres from eight vertebrate tissues and cell cultures, as well as two tissues from an invertebrate. All telomeres have substantial tracts of $(TTAGGG)_n$ comprising 0.01–0.2% of the genome. All telomeres are long (20–100 kb), except for those of sea urchin, human, and some chicken chromosomes, which are 3–10 kb in length. All of the animal telomeres contained nucleosome arrays, consistent with the original hypothesis. The telomere repeat lengths vary from 151 to 205 bp, seemingly uncorrelated with telomere size, regularity of nucleosome spacing, species, or state of differentiation but surprisingly correlated with the repeat of bulk chromatin within the same cells. The telomere nucleosomes were consistently ≈40 bp smaller than bulk nucleosomes. Thus, animal telomeres have highly conserved sequences and unusually short nucleosomes with cell-specific structure.**

Telomeres are functionally and structurally distinct structures at the ends of eukaryotic chromosomes that are essential for chromosome stability and also seem important for the expression of adjacent genes, spatial arrangement of chromosomes in nuclei, and initiation of chromosome pairing during meiosis (1, 2). In protozoa and fungi the telomere DNA tracts are very short (18–600 bp), contain a 3′ G-rich single-stranded tail, and are bound to nonhistone proteins, in contrast to the rest of the genome, in which the DNA and histone proteins are organized into nucleosome arrays (3). The telomeres of animals and plants are substantially longer (2–100 kb) and less well characterized (4–7). The length of telomeres from human somatic cells is directly related to the mitotic history of the cells, with an average shortening of ≈100 bp per division (8, 9). As telomeres reach a critical length, chromosomes seem to become unstable (10). Only immortal cells from lower eukaryotes and germline and tumor cells from higher eukaryotes have telomeres of constant length, apparently stabilized by the enzyme telomerase, which is able to add telomere sequences to the 3′ termini (11).

We recently characterized the nucleoprotein structure of rat telomeres by nuclease and sedimentation analyses (12). Micrococcal nuclease (MNase) studies revealed very regular arrays of nucleosomes spaced by 157 bp on the telomeres, representing the shortest nucleosomes found in animals and plants. DNase I digestion patterns and electrophoretic mobilities of the telomere nucleosomes were identical to those of bulk chromatin, suggesting that the protein composition of the telomere core particles was very similar to that of bulk nucleosomes. Gel electrophoresis of nucleoproteins indicated that telomere core particles did not bind histone H1, yet sedimentation analysis showed that the mononucleosomes and oligonucleosomes of telomere and bulk chromatin cosediment at low ionic strength and are sensitive to removal of H1. Several of these experiments have been replicated with human and mouse cell lines, giving the same results (13).

Studies of the origin and nature of these telomere-specific nucleosomes might give insight into the general process of nucleosome assembly and into the roles of telomeres in chromosome stability and cellular senescence. In this paper we address the question of telomere DNA and nucleoprotein structure in organisms representing the vertebrate classes Mammalia, Reptilia, Aves, Amphibia, and Pisces, as well as the invertebrate class Echinodea. The results support the hypothesis that animal cells have highly conserved telomere DNA sequences of $(TTAGGG)_n$ organized largely into short nucleosomes of variable length, usually ≈40 bp less than nucleosomes of bulk chromatin. In addition, the distinctness of the nucleosomal ladder appears to be correlated with the length of the telomere tracts, suggesting that short telomeres might be less homogeneous than long telomeres.

MATERIALS AND METHODS

Cell and Nuclear Isolation. Rat (*Rattus norvegicus*) and mouse (*Mus musculus*) hepatocytes were obtained from freshly killed animals. Chicken (*Gallus domesticus*), turtle (*Pseudemys scripta*), mud puppy (*Necturus maculosus*), and trout (*Oncorhynchus mykiss*) erythrocytes were obtained from fresh whole blood. Human neutrophils were separated from fresh blood by centrifugal elutriation (14). JEG-3 human cells and B103 rat brain neuronal cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum and released by trypsin treatment. Sea urchin (*Strongylocentrotus purpuratus*) gametes were collected in synthetic sea water. Embryos were collected 36 hr after fertilization, as described (15).

Nuclei from liver tissues were isolated as described (12). Tissue culture and fresh blood cells were washed twice by centrifugation at 800 × *g* with phosphate-buffered saline, resuspended in a small volume, and washed twice with buffer A (15 mM NaCl/15 mM Tris·HCl, pH 7.4/60 mM KCl/3 mM $MgCl_2$). Cells were resuspended in 10 volumes of lysis buffer (buffer A with 0.1% digitonin, 1 mM phenylmethylsulfonyl fluoride, 6 μM leupeptin, and 1 mM iodoacetate) at 4°C and immediately centrifuged at 800 × *g* at 4°C for 10 min. Pelleted nuclei were washed twice with lysis buffer without detergent. Nuclei from sea urchin sperm and embryos were isolated as described (15, 16).

Enzymatic Digestion. To measure the length of telomere tracts, DNA was isolated from nuclei (12) and digested with 5

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Abbreviations: MNase, micrococcal nuclease; TELG4, oligonucleotide $(TTAGGG)_4$.

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units of restriction enzyme *Hae* III or *Hin*fI per 1 μg of DNA for 16 hr at 37°C. Terminal location of the $(TTAGGG)_n$ sequences was determined by testing for progressive digestion with exonuclease BAL-31 (4) or from studies of *in situ* hybridization (17, 18). MNase digestions were carried out at 37°C for 0.5, 1, 2, 5, and 10 min with 2 and 5 units enzyme per optical density unit of nuclei (12).

Electrophoretic Analysis and Hybridization. To measure the molecular size of the restriction fragments containing telomere DNA, the *Hin*fI- or *Hae* III-digested DNA was electrophoresed by pulsed-field inversion in 1% agarose at 8 V/cm and 4°C for 13 hr and electrophoretically transferred to a Zeta-Probe GT (Bio-Rad) membrane (12). DNA was denatured and crosslinked to the membrane by alkaline treatment, prehybridized, and hybridized with a mixture of ^{32}P-labeled oligonucleotide $(TTAGGG)_4$ (TELG4) and marker DNA probes, labeled by use of T4 kinase and random priming, respectively (12). Increase of the final wash temperature from 50°C to 55°C decreased the hybridization signals by a factor of 10 but did not change the relative strength of signals from different organisms, showing that the hybridization was specific to $(TTAGGG)_n$ in all cases. Hybridization was quantitated with a Molecular Dynamics 400A PhosphorImager and IMAGEQUANT software. Percentage of telomere DNA (telomere DNA/bulk DNA) was estimated by quantitation of the sample hybridization signals (normalized to the amount of bulk DNA) and comparison with the normalized hybridization signal of pHuR93 plasmid DNA (American Type Culture Collection) which contains 240 bp of telomere sequence.

MNase cleavage patterns of interphase nuclei were assayed by electrophoresis in 1.5% agarose followed by staining with ethidium bromide and blot/hybridization with TELG4. The same results were found with oligonucleotide $(CCCTAA)_4$. Gels and autoradiograms were quantitated by CCD fluorography and PhosphorImager autoradiography (12). Repeat lengths were determined from the slopes (by linear regression) of graphs of fragment size vs. fragment number for short digestion times (12).

RESULTS

Size, Abundance, and Location of the Telomere Satellite DNA of Vertebrates and Invertebrates. We determined the size, abundance, and location of the $(TTAGGG)_n$ satellites. The restriction enzyme *Hae* III released intact telomere satellite DNA, which was detected by stringent hybridization with TELG4. Fig. 1 and Table 1 show that the average telomere lengths are variable (3–9 kb in human, sea urchin, and some chicken chromosomes; 100 kb in mud puppy and other chicken chromosomes). All telomeres except those from mud puppy erythrocytes have the typical broad distribution in

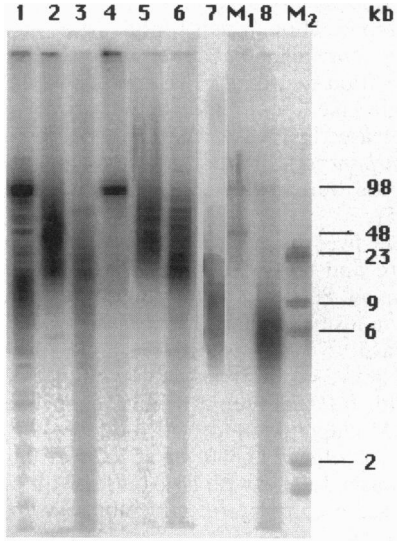

FIG. 1. Pulsed-field inversion electrophoretic analysis of telomere DNA size. Lane 1, chicken erythrocytes; lane 2, turtle erythrocytes; lane 3, trout erythrocytes; lane 4, mud puppy erythrocytes; lane 5, rat liver; lane 6, rat cultured cells; lane 7, human neutrophils; lane 8, sea urchin embryos. Size markers were phage λ DNA and a λ *Hin*dIII digest (lanes M_1 and M_2, respectively).

Table 1. Characteristics of $(TTAGGG)_n$ tracts in various organisms and cell types

Source	Length (mode), kb	Abundance, %
Human neutrophils	9	0.02
Rat hepatocytes	50	0.1
Chicken erythrocytes	10,100	0.2
Turtle erythrocytes	50	0.2
Mud puppy erythrocytes	100	0.06
Trout erythrocytes	20	0.06
Sea urchin embryos	6	0.06
B103 rat cells	40	0.08
JEG3 human cells	3	0.01

Average telomere tract lengths were determined by analysis of lanes 1–8 in Fig. 1. All telomere sequences were confirmed to be terminal by BAL-31 exonuclease digestion or *in situ* hybridization, except for trout.

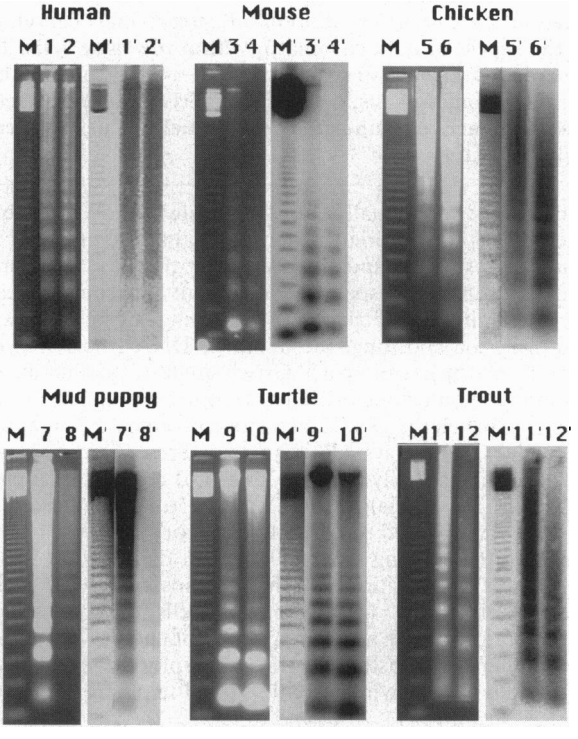

FIG. 2. Electrophoresis of bulk and telomere chromatin from vertebrate cell nuclei digested with MNase. Lanes labeled with numerals are from ethidium bromide gels; lanes labeled with primed numerals are the same lanes after transfer to filters, hybridization with TELG4, and autoradiography. Lanes M, 123-bp ladder; lanes 1 and 2, human neutrophil nuclei digested 1 and 2 min; lanes 3 and 4, mouse liver nuclei digested 2 and 5 min; lanes 5 and 6, chicken erythrocyte nuclei digested 1 and 2 min; lanes 7 and 8, mud puppy erythrocyte nuclei digested 1 and 2 min; lanes 9 and 10, turtle erythrocyte nuclei digested 2 and 5 min; lanes 11 and 12, trout erythrocyte nuclei digested 1 and 2 min. Lanes for human and rat cultured cells are not shown. The lanes shown were selected to be adjacent to marker lanes and to have visible bands in the high-contrast, low molecular weight region. Contrast on marker autoradiogram lanes was enhanced for presentation.

length. Chicken terminal fragments have a bimodal length distribution. The diversity in the patterns suggests that the mechanisms that control the length of telomeres might be different in different cells and on different chromosomes.

Quantitation of the hybridization signals (Table 1) shows that the abundance of telomere DNA varies from 0.01% to 0.2%. As expected, there is a general correlation between telomere size and abundance (compare human, rat, chicken, turtle, and fish). Sea urchin had the highest ratio of telomere abundance to telomere length, consistent with the large number of chromosomes (19).

Restriction enzyme-digested chicken and rat DNAs in Fig. 1 have noticeable high-resolution bands [most visible in overexposures and previously noted for mouse (5, 7)] indicative of small amounts of interstitial telomere sequences, despite *in situ* hybridization evidence that the vast majority of the telomere sequences are at the ends of the chromosomes (17, 20). The lack of susceptibility of high molecular weight telomere DNA to multiple restriction enzymes [*Hae* III and *Hin*fI in this study and eight restriction enzymes in our earlier study (12)] and the strong hybridization of the telomere oligonucleotides under stringent conditions make it seem likely that most telomere tracts are homogeneous.

In situ hybridization and BAL-31 digestion studies have shown that the human, rat, mouse, and chicken satellites are primarily at the ends of chromosomes (4–7, 12, 17, 18, 20). BAL-31 digestion of chicken, turtle, mud puppy, and sea urchin telomere DNA showed a gradual decrease of size and hybridization signal (data not shown), indicating terminal telomere sites. Trout telomere fragments were not trimmed by BAL-31 exonuclease in two independent trials but had the broad size distribution expected for a terminal location. BAL-31 nuclease did not decrease the size of the ethidium bromide stained bulk DNA restriction fragments of all tissues studied (data not shown), confirming BAL-31 specificity for termini.

Nucleosomal Structure of Telomeres. MNase digestion of vertebrate nuclei and electrophoresis of the DNA revealed a repeating nucleosomal substructure on both bulk and telomere sequences, of similar sensitivities to digestion (Fig. 2). Vertebrate telomeres appeared to be constructed primarily of repeating subunits, characterized by monomers of ≈145 bp and multimeric species separated by 150–167 bp, based on the slope of the linear regression analysis of the graphs of band number vs. molecular weight. Surprisingly, the telomere repeat length was not the same in every cell, as we had expected on the basis of conservation of the telomere DNA sequence.

Further analysis was done on sea urchin telomeres, because the nucleosome repeat lengths are much longer than those from the other cells. MNase digestion of 36-hr embryos and sperm (Fig. 3*A*) shows that echinoderm telomere nucleosomes are longer than those of vertebrates, and there is a correlation between the bulk and telomere repeats (the length ratio telomere vs. bulk repeats is 174:213 for embryos and 205:247 for sperm). Purified DNA from the MNase-treated nuclei was digested with *Hin*fI or *Hae* III, which obliterated the nucleosome ladder of bulk but not telomere chromatin (Fig. 3*B*), showing that the long nucleosome repeat is not an artifact due to a mixture of telomere and nontelomere sequences. The terminal location of these echinoderm telomere satellites was confirmed by BAL-31 digestion, which did not affect the 6.5-kb early histone gene repeat, but dramatically reduced the size and amount of telomere DNA (Fig. 3*C*). These data demonstrate that vertebrates and invertebrates have significant amounts of telomere-specific chromatin at the termini of the chromosomes.

Qualitatively, the distinctness of the nucleosome ladder for the various animals seems to be correlated with the length of the telomere tracts. A similar effect has been observed recently in immortal human cell lines (13). The least distinct patterns were found from the shortest telomeres, from human (Fig. 2) and sea urchin (Fig. 3). The implications of these observations will be discussed later.

Fig. 4 compares the repeat lengths for bulk and telomere chromatin from all animals we have analyzed. The telomere repeat lengths are 27–47 bp shorter than the bulk repeat lengths. Comparing the results from the primary cells, the slope of the linear regression is 1.03 ± 0.09 (correlation coefficient, 0.97), with an average difference between the telomere and bulk chromatin of 41.2 bp. The cell lines have

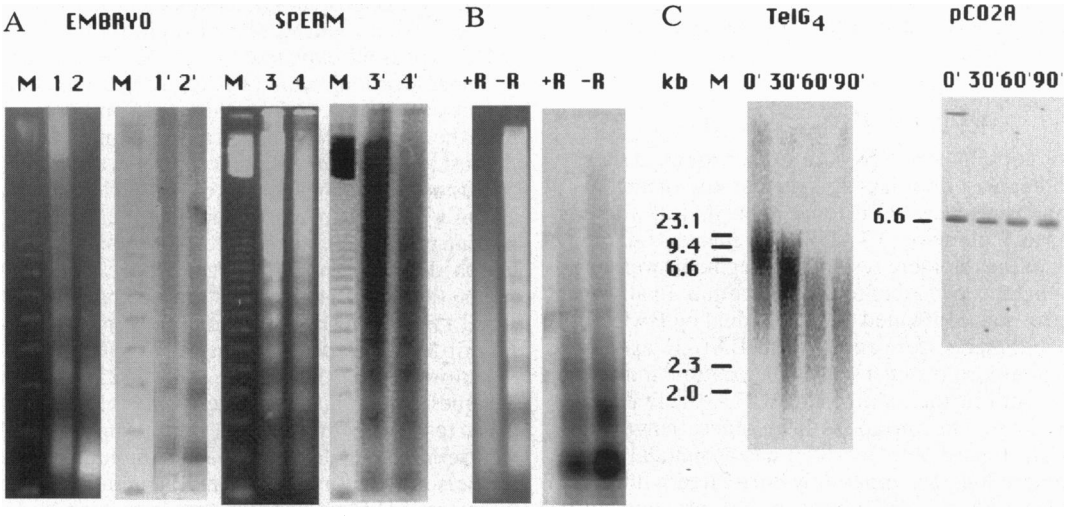

FIG. 3. Electrophoretic analysis of sea urchin. Numerals denote ethidium bromide staining; primed numerals denote filter hybridization of the same gel lanes with TELG4. (*A*) MNase digestion ladders. Lanes M, 123-bp ladder; lanes 1 and 2, embryo nuclei digested 1 and 5 min; lanes 3 and 4, sperm nuclei digested 5 and 10 min. MNase trimming of the long linkers is evident. (*B*) Test of the sequence purity of the telomere nucleosome arrays. Sperm nuclei were digested 10 min with MNase and the DNA was electrophoresed with (lanes +R) and without (lanes −R) *Hin*fI restriction. The nucleosome ladder from bulk DNA was obliterated due to internal restriction; the nucleosome repeat from telomere DNA was unaltered. Restriction with *Hin*dIII gave the same result (data not shown). (*C*) Terminal location of the sperm telomere DNA. Agarose-embedded nuclei were digested with BAL-31 exonuclease for 30, 60, and 90 min. Purified DNA was digested with *Hin*fI and probed with TELG4 (*Left*) or digested with *Xho* I and probed with pCO2A, (a plasmid containing the complete 6.5-kb early histone gene repeat) (*Right*), producing telomere and histone gene fragments of comparable sizes. Size markers (M) were *Hin*dIII fragments of phage λ DNA. Telomere DNA was trimmed by BAL-31, whereas the histone genes were resistant, confirming the terminal location of telomere DNA.

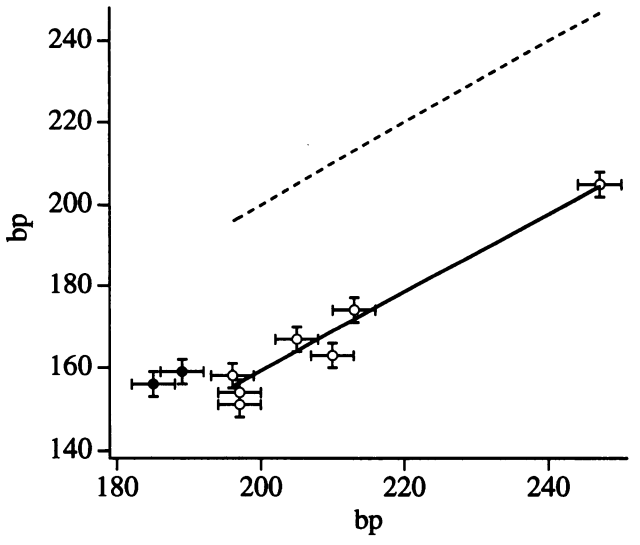

FIG. 4. Comparison of the nucleosome repeat lengths of telomere and bulk chromatin. Abscissa, repeat length of bulk chromatin determined from ethidium bromide staining; ordinate, repeat length from telomere chromatin determined from the same gel lanes after transfer to filter for hybridization with TELG4. Data points are from all the tissues studied, with estimated uncertainties. ○, Primary cells; ●, cell lines. Solid line is a linear regression on the data from all primary cells; dashed line is consistent with the hypothesis that the nucleosome repeat lengths in telomeres and bulk are identical. The length (bp) ratios of nucleosome repeats (telomere/bulk) of various samples were as follows: human neutrophils, 151:198; mud puppy erythrocytes, 154:197; rat hepatocytes, 157:197; mouse hepatocytes, 158:196; turtle erythrocytes, 158:196; chicken erythrocytes, 167:204; trout erythrocytes, 163:210; B103 rat cultured cells, 158:185; JEG-3 human cultured cells, 159:189; sea urchin embryos, 174:213; sea urchin sperm, 205:247.

slightly greater than expected telomere repeats, possibly due to the anomalously short repeat lengths of cultured cells (3), a lower limit to the telomere repeat, or different telomere structure in the rapidly growing immortal cells. The ≈40-bp difference between bulk and telomere nucleosomes is an example of locus-specific chromatin structure and suggests a conserved difference between assembly of nucleosomes on bulk and telomere DNA.

DISCUSSION

The characteristics of telomere DNA are similar for all classes of vertebrates and at least one class of invertebrates. Stringent hybridization conditions were used to confirm that all these animals had the same telomere $(TTAGGG)_n$ repeat as human and that the bulk of the telomere restriction fragments were of heterogeneous length, expected of terminal location. In all but one case the repeat was confirmed to be terminal by BAL-31 digestion. Trout telomeres were resistant to BAL-31 exonuclease, but we cannot rule out a terminal location for the telomere satellite until further controls of BAL-31 activity or cytogenetics are done. The variations in telomere length in various animals are remarkable. Chicken has a bimodal distribution of telomere lengths, apparently correlated with cytogenetic evidence of large differences in the amounts of telomere satellite on different chromosomes (20).

The telomeres of all the animals we studied contain arrays of nucleoprotein subunits similar to the well-characterized nucleosomes present on rat telomeres (12). This supports our original hypothesis that animal telomeres have nucleosomes.

The conserved features of telomere-specific DNA chromatin structure in a wide variety of animals suggest common functions and origins. The origin of the short repeat length might be related to the sequence or the terminal location of the $(TTAGGG)_n$ satellite. Contrary to expectation, the telomere nucleosome repeat lengths are not conserved but are ≈40 bp shorter than those of bulk chromatin. The absence of a conserved spacing for the telomere nucleosomes seems to rule out the simplest explanation, that the repeat length is dictated solely by DNA sequence. The differences in repeat length seem uncorrelated with (*i*) length of the telomere tracts (sea urchin and human have the smallest telomeres but extremely different repeat lengths; rat and human have the same repeat lengths but extremely different telomere lengths), (*ii*) distinctness of the nucleosome ladder (rat and human have similar repeats but extremely different distinctness), (*iii*) species (sea urchin embryos and sperm have very different repeats), or (*iv*) state of differentiation (immortal cell lines, neutrophils, some erythrocytes, and hepatocytes have similar repeats). This difference between bulk and telomere chromatin could be the result of differences in the mechanism of nucleosome assembly, the rate of replication, the topological constraint or physical location, or nonhistone proteins.

One explanation for the origin of the short telomere nucleosome repeat is suggested by data that bulk chromatin is initially assembled in a short repeat that matures into a longer repeat, perhaps resulting from histone modifications and interaction with nonhistone proteins (21, 22). *In vitro* reconstitution of chromatin in *Xenopus,* and *Drosophila* tissue culture extracts indicates that several factors influence nucleosome spacing in a stepwise fashion (21–23). Binding of either phosphorylated high-mobility-group protein 14/17 (24, 25) or histone H1 (26–28) seems to increase the spacing of nascent nucleosomes by 10–20 bp. If telomeres were deficient in one or more of these maturation steps (e.g., due to reduced affinity of the DNA for certain assembly components or to interference by telomere-specific proteins) incomplete maturation could result in a repeat shorter than, but related to, the bulk nucleosome repeat. Study of the differences between chromatin assembled *in vitro* on bulk and telomere DNA might shed light upon the mechanism of nucleosome assembly and the regulation of repeat length.

It has been observed that reduction in the replication rate can reduce nucleosome spacing (29). Therefore, more slowly replicating regions of the genome might accumulate a higher density of nucleosomes. Perhaps the short telomere repeat is the result of a reduced rate of replication on telomeres.

Other possible explanations for the short spacing involve the terminal location of the $(TTAGGG)_n$ tracts. Lacking normal matrix-attachment regions, these tracts might not be organized into the topologically constrained domains characteristic of the rest of the genome (30). Perhaps the short spacing is caused by postassembly compaction of the nucleosomes due to interaction with nuclear matrix or to cooperative binding of nonhistone proteins at the tips of chromosomes. If the short repeat length depends upon the terminal location of the telomere tracts, then an organism that has extensive amounts of interstitial $(TTAGGG)_n$ tracts should exhibit a bimodal distribution of MNase repeat lengths.

Unfortunately, the MNase digestion results do not answer any questions about non-nucleosomal telomere structure, contrary to the assumption of Tommerup *et al.* (13) that the diffuse MNase ladders from short human telomeres indicate the presence of non-nucleosomal structure at the distal ends of telomeres. Diffuse ladders can be attributed to irregular nucleosome spacing, instability of core particles to MNase, or binding of nonhistone proteins. For example, the well-known sequence variations at the proximal end of human telomeres (31) could lead to heterogeneity in nucleosome spacing, especially in cells with eroded telomere ends, such as those studied by Tommerup *et al.* (13). Cases of irregular nucleosome spacing in nontelomere DNA are well documented. For example, indistinct ladders are usually found after chromatin reconstitution with purified histones or in cell-free extracts, as

well as in native simian virus 40 chromatin and transcriptionally active genes (26, 27, 32, 33). Conversely, qualitatively intact nucleosome ladders can be found even in the presence of substantial amounts of non-nucleosomal structure. For example, mouse rDNA gives qualitatively normal MNase ladders, despite the fact that >50% of the gene copies do not have nucleosomes (34). Thus, there is no direct evidence of nonhistone proteins bound to the telomeres, and we can conclude only that all telomeres that we have studied have some regions consisting of regularly spaced nucleosomes, as well as variable amounts of irregularly spaced nucleosomes or non-nucleosomal structures that are digested at approximately the same rate as nucleosomes.

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